Specific Immune Response Induced by Immunization with Self Epidermal Growth Factor Receptor-Extracellular Domain

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We propose a new approach of active specific immunotherapy targeting EGFR, which consist in the immunization with the extracellular domain (ECD) of self EGFR in adjuvants to circumvent the tolerance to self EGFR inducing an immune response with biological effects over EGFR+ tumor cells.

Our results suggest that ECD-Her1/VSSP may be used as a vaccine for patients with EGFR+ tumors.

Key words: EGFR, self immunization, cancer therapy

Abbreviations: ECD-MEGFR (extracellular domain of epidermal growth factor receptor)

VSSP (Very Small Sized Proteoliposomes from *Neisseria meningitides)*

ABSTRACT:

The Epidermal Growth Factor Receptor (EGFR) plays a central role in regulating neoplastic processes. The EGFR overexpression in many human epithelial tumors has been correlated with disease progression and bad prognosis. Passive EGFR-directed immunotherapy but not active specific approaches have already been introduced in medical Oncology practice. Then we wonder if mice immunization with the extracellular domain of murine EGFR (ECD-mEGFR) in adjuvants can circumvent the tolerance to self EGFR inducing an immune response with biological effects over EGFR+ tumor cells. The present study demonstrated that despite mEGFR expression in thymus, strong DTH response was induced by the immunization with the ECD-mEGFR in Complete Freund Adjuvant (CFA). Besides, the immunization with the ECD-mEGFR in CFA or Very Small Sized Proteoliposomes from *Neisseria meningitidis* (VSSP) induced high specific IgG titers, with IgG2a and IgG2b isotypes. In addition, mice were immunized with the ECD of human EGFR (ECD-Her1) to compare the self-recognition induced by self and non-self immunization. The immunization with ECD-Her1 in VSSP induced high specific IgG titers but low crossreaction with the ECD-mEGFR. Sera from mice immunized with ECD-mEGFR/VSSP or ECD-Her1/VSSP not only recognized by FACS EGFR+ murine or human tumor cell lines respectively but also inhibits the *in vitro* growth of those cells. A complement-independent cytotoxic effect of immune sera from self and non-self immunization is also documented. Non reproductive side effects were produced by

immunization. Our results suggest that ECD-Her1/VSSP may be used as a vaccine for patients with EGFR+ tumors.

INTRODUCTION

The epidermal growth factor receptor (EGFR) belongs to the erbB family of four closely related cell membrane receptors, also known as the Type I Receptor Tyrosine Kinase family: EGFR or HER1/erbB1, first to be molecularly cloned (1), HER2/erbB2, HER3/erbB3 and HER4/erbB4. 'The four receptors consist of an extracellular ligand-binding domain (ECD), a transmembrane domain, and an intracellular domain with tyrosine kinase activity for signal transduction. EGFR plays a central role in regulating both development and neoplastic processes. Binding of their specific ligands, such as epidermal growth factor (EGF) or transforming growth factor-α (TGF-α) among others, induces receptor activation, modulation of cell proliferation and differentiation in normal tissues and tumors. Although expressed in nonmalignant cells, the EGFR can be found overexpressed or mutated in many human epithelial tumors such as breast (2,3), lung (4), prostate (5,6) head and neck (7), colorectal (8), pancreatic (9), bladder (10), vulva and ovarian tumors (11). This overexpression has been correlated with disease progression and poor prognosis (2,12,13). Activation of the EGFR signaling pathway in cancer cells have shown to enhance cell proliferation, angiogenesis, tumor promotion and metastasis, and to decrease apoptosis. The potential of EGFR-targeted therapies for cancer treatment has increased the development of different passive agents. Passive immunotherapy with specific monoclonal antibodies (MAb) (14,15) and treatment with tyrosine kinase inhibitor drugs such as Iressa (16,17) and Tarceva (18), are currently undergoing clinical trials with

promising results or are commercially available. On the other hand, active immunotherapy strategies to block the EGF from binding to its receptor are being clinically tested by vaccinating patients with EGF coupled to P64k recombinant protein from *Neisseria meningitidis*l (19).

In addition EGFR-based active specific immunotherapy may be an alternative and complementary approach for the treatment of epithelial tumors, provided that induction of an immune response against self EGFR is feasible. Preclinical studies of both DNA vaccine based on ECD-xenogenic EGFR and dendritic cell pulsed with ECD-self EGFR have been recently published and demonstrate the validity of active immunotherapy (20- [1]). Here, we propose an approach based on vaccination with the protein of the ECD-mEGFR, thus exploring the possibility of circumventing the tolerance to self EGFR. We constructed plasmids DNA encoding the ECD of murine EGFR which was stably transfected in mammalian cells. The corresponding recombinant protein was used for vaccination protocols. Besides, we expressed the ECD-Her1 to compare the self-recognition induced by self and non-self immunization, and to evaluate the specific immune response over EGFR+ human tumor cells.

The present study demonstrates that ECD-mEGFR in FA generated a strong DTH response, and that using both, Freund Adjuvant and Very Small Size Proteoliposomes from *Neisseria meningitidis* (VSSP), can be induced a specific humoral immune response with high IgG titers with a TH1 associated isotype. In addition, the immune sera recognized EGFR+ cells, inhibited tumor cells proliferation *in vitro* and mediated complement independent citotoxicity of those cells. Besides, the immunization with ECD-Her1 induced high IgG titers, and the immune sera recognize EGFR+ human tumor cell lines, inhibited

their growth in vitro, and mediated complement independent citotoxicity of those cells. But the specific immune response against Her1 presented low crossreaction with mEGFR. Consequently, our results suggest that immunization with ECD-Her1 in VSSP may provide a novel strategy for cancer immunotherapy for patients with EGFR+ tumors.

MATHERIAL AND METHODS

Construction of the expression vector encoding ECD-mEGFR and ECD-Her1.

DNA encoding the extracellular domain of murine EGFR was amplified by PCR using total cDNA from mouse liver as template. The sense primer, 5´-CGGAATTCCTCTCCCGGTCAGAGATGCGAC-3´ includes EcoRI excision site, the initiation codon and 4 bp from signal sequence of EGFR. The antisense primer, 5´-CGGGATCCTCAAGATGGTATCTTTGGCCCAGATG-3´ is complementary to bp 1978- 2000 in 3' region and contains a stop codon (double underline) and BamHI excision site (single underline). The PCR product, a 1.9 kb fragment, was cloned into EcoRI/BamHI sites of pBluescript KS^+ vector. The fragment encoding for ECD-MEGFR was recovered using HindIII/BamHI enzymes and cloned into pcDNA3 expression vector (Invitrogene, San Diego, CA, USA) generating the plasmid ECD-MEGFR/pcDNA3.

DNA encoding extracellular domain of human EGFR (HER1-ECD) was amplified by PCR using HER1Δ533/pRK5 plasmid as template. The sense primer 5´-GGGGTACCCTTCGGGGAGCAGCGATGCGA-3´ includes KpnI excision size (underline), the initiation codon ATG and 3 bp from the signal sequence of HER1. The antisense primer, 5´-GCTCTAGATCAGGACGGGATCTTAGGCCCA-3´ is

complementary to bp 2103-2123 in the 3' region, and contains a stop codon (double underline) and XbaI excision site (single underline). The PCR product, a 1.9-kb fragment, was cloned into KpnI/XbaI sites of pcDNA 3-expression vector to generate the plasmid HER1-ECD/pcDNA3.

The sequences of the ECD-mEGFR and ECD-Her1 were confirmed by dideoxy nucleotide sequencing analysis to be identical with those previously reported (21,22). All enzymes were supplied by Boehringer Mannheim, Penzberg, Germany.

Cell lines

Ehrlich Ascites Tumor (EAT, ECACC No 87032503), 3LLD122 murine metastatic variant of Lewis lung carcinoma (23), murine thymoma EL4 (ATCC TIB-39), Human embryonic kidney (HEK293, ATCC CRL-1573), Human epidermoid carcinoma A431 (ATCC CRL-1555) and Human lung adenocarcinome $H125^2$ cell lines were grown in DMEM (Gibco, USA) suplemented with 10% fetal calf serum (FCS) (Hyclone, Utah, USA), 2mM L-glutamine, 1mM sodium pyruvate, penicillin 100 U/mL / and streptomycin 100 μg/mL (Life Tchnologies, Grand Island, NY). HEK293 transfectant was adapted to growth in HyQ PF 293 special protein free medium (Hyclone, USA).

Generation of HEK293 transfectant

HEK293 cells were grown in 6 well plates ($1.75x10^5$ cell/mL) and 8 hours later were transfected with 4 μg of ECD-mEGFR/pcDNA3 or ECD-Her1/pcDNA3 plasmids, using the calcium phosphate transfection system. Plates were incubated overnight at 3% CO2, and then at 5% CO2. Transfected cells were selected in medium containing 1,000 μg/mL of G418 (Geneticin, Sigma, USA) starting 48 hours after transfection for the

generation of ECD-mEGFR/HEK293 and ECD-Her1/HEK293 stable cell lines. Mock transfection (with pcDNA3 vector) was used as a negative control.

Lectin precipitation and immunoprecipitation of recombinant proteins

Supernatant from ECD-mEGFR/HEK293 or ECD-Her1/HEK293 transfectant cultures (2 mL) were mixed with 10 μL of lectin-agarose (lectin from *Triticum Vulgaris*, Sigma, St Louis, USA) or 1 μg of MAb R3 (MAb specific for the extracellular domain of human EGFR, CIM, Cuba) plus 20 μl of protein A-sepharose (Amershan Pharmacia Biotech, Uppsala, Sweeden), respectively. Samples left overnight at 4°C with gentle shaking were centrifuged 1 min at 11000 g, and the precipitated recombinant proteins were separated on SDS-PAGE 7.5%, and visualized by silver staining.

Purification of ECD-mEGFR and ECD-Her1

Recombinant proteins were purified from a confluent culture of the transfectant by affinity chromatography. EAH-sepharose 4B (Amershan Pharmacia Biotech, USA) was covalently coupled to human recombinant EGF (hrEGF) (Center of Genetic Engineering and Biotechnology (CIGB), Cuba) for ECD-mEGFR purification or to MAb R3 for ECD-Her1 purification. The equilibration and washing steps were performed with PBS/NaCl 1M pH 7.0 and the elution with Glycine 0.2*M* pH 2.8. The proteins purity was measured by densitometry, using a personal densitometer SI (Amersham Pharmacia Biotech, USA) and Imag Quant Software. Protein concentration was assayed by Lowry method (24).

RT-PCR

Total RNA was isolated from Balb/c or C57BL/6 thymus using TRIZOL Reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. The reverse transcription and polymerase chain reaction (RT-PCR) was performed using the SUPERSCRIPT™ One-Step RT-PCR System according to the manufacturer's instructions. The primers used for EGF-R and β-actin were designed from the published sequences (25) . After PCR amplification, 10 μL of the RT-PCR products were separated by electrophoresis on 1.5% agarose gel and visualized with Ethidium bromide. PCR amplification was run for RNAs reverse transcribed in parallel with RNAs without reverse transcriptase and any contaminating DNA was seen in the samples. Total RNAs from murine thymoma EL4 was used as negative control.

Mice and Immunization protocols

Female C57BL/6 mice, aged 8-12 weeks old, were purchased from the National Center of Laboratory Animals Production (CENPALAB, Havana, Cuba). All mice were kept under pathogen-free conditions. The animal experiments were approved by the Center of Molecular Immunology's Institutional Animal Care and Use Committee's normative (CIM, Havana, Cuba).

Mice (n=5 for DTH study and n=10 for humoral response studies) were immunized with 50 μg of ECD-mEGFR or 50 μg of ECD-Her1. The adjuvants used were FA, complete for the first immunization and incomplete for the rest (Sigma, USA) and VSSP, obtained from the combination of the outer membrane proteins of *Neisseria meningitidis* with GM3 ganglioside, in a water in oil (Montanide ISA 51, Seppic, Paris, France) emulsion (26).

Immunizations were made, subcutaneously (sc) for FA adjuvated or intramuscularly (im) for VSSP adjuvated preparations, on days 0, 14 (for DTH study) and 0, 14, 28, and 42 (for humoral response studies). Sera were extracted on days 0, 21, 35, 56. Control groups received PBS/FA or PBS/VSSP.

DTH test

Seven days later of the last immunization, mice were sensitized by intradermal injection with 50 μg of ECD-MEGFR in 50 μL of PBS in the right hind foot pad and received the same volume of PBS in the left foot pad. After 48 hours the volume of inflammation (swelling of the mice foot) was measured using a plethysmometer (Ugo Basile, VA, Italy). Mice immunized with 100 μg of Keyhole limpet hemocyanin (KLH), (Sigma, Aldrich, USA) in FA and sensitized with KLH in PBS, were used as a positive control. Mice injected with PBS/FA and sensitized with ECD-MEGFR were used as negative control.

Enzyme immunoassay

Microtiter plates (High binding, Costar, USA) were coated with 10 μg/mL of ECD-mEGFR or ECD-Her1 in carbonate buffer, 0.1 *M*, pH 9.6, and incubated overnight at 4°C. Plates were blocked with 5% calf serum in PBS/Tween-20, and sera dilutions in duplicate, from immunized mice (n=10), were incubated 1 hour at 37°C. Alkaline phosphatase conjugated goat anti-mouse IgG antibody (Sigma, USA) was added and incubated 1 hour at 37°C. After addition of P-nitrophenylphospate (1 mg/mL) (Sigma, USA) the OD was measured at 405 nm using a microwell system reader (Organon Teknica Inc., Salzburg, Austria). All washes were made with PBS/Tween-20.

Determination of IgG isotype profile in the sera of immunized mice (n=5) was performed using as secondary isotype-specific biotinylated rat antimouse IgG1, IgG2a, IgG2b, or IgG3 antibodies (PharMingen, USA). Optimal secondary antibody dilutions were established by ELISA with the MAbs 14F7 (IgG1 specific for NGcGM3), T3 (IgG2a specific for CD3) and T4 (IgG2b specific for CD4) (CIM, Cuba) and R24 (IgG3 specific for GD3) (kindly provided by Dr. Philip O. Livingston, Memorial Sloan Kettering Cancer Center, NY, USA).

ELISPOT

Specific spot forming cells (SFC) in inguinal Lymph nodes (LN) from mice immunized with ECD-Her1/VSSP were tested by Enzyme linked immunospot (ELISPOT) assay previously described,[3] with some modifications. Maxisorp 96 well plates (Nunc, USA) were coated with 10 μg/ml of HER1-ECD or mEGFR-ECD, in 50 μl carbonate buffer (pH 9.8) at 4°C, overnight. After blocking with 5% BSA in PBS, different dilutions of pooled LN cells were incubated in triplicate 6 hr at 37°C in a 5% CO_2 incubator. Antibodies secreted by individual cells were revealed as spots by the stepwise addition of 1,5 μg/ml of alkaline phosphatase conjugated goat anti mouse IgG (Fcγ) or anti mouse IgM (Fcμ) antibody (Jackson Immunoresearch laboratories Inc, West Grove, PA) and later the addition of chromogen substrate, 1mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP, Sigma, San Louis, MO) in 0.1 M AMP buffer pH 10.5 containing 0.6% agarose. Plates were incubated at 4°C, overnight, and the results were scored the next day by counting the number of specific spot forming cells in a stereoscopic microscope. LN cells from mice injected with PBS/FA were used as negative control.

FACS Analysis for EGFR recognition

Cells were stained with sera from immunized mice, diluted 1/200 followed by FITC-goat antimouse IgG (Jakson, Immunoresearch laboratories Inc, West Grove, PA). Next up to 10,000 cells were acquired using a FACScalibur flow cytometer and analyzed using the CellQuest software (Beckton Dickinson, San Jose, CA, USA). PCR and MAb 7A7 (27), which is specific for ECD of murine EGFR, were used to confirm the expression of EGFR in murine cells. The expression of human EGFR in human cells was confirmed with the MAb R3. EL4 murine cell line and human lymphocytes were used as negative control cells.

Growth Assay

Flat bottomed 96-well microculture plates were seeded with 10^4 cells in 100 μL/well and grown in DMEM suplemented with 1% FCS in presence of dilutions of sera. After 48 hours of incubation at 5% of CO2, viability of cells was measured using a modified colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazodium bromide) assay (28,29). The medium was replaced by 100 μL/well of MTT (1 mg/mL) and plates were incubated under culture conditions 4hours. The formazan crystals were dissolved by addition of 100 μL/well of Dimethyl sulphoxide followed by incubation 30 minutes at 37°C. The absorbance (OD) was measured at 540 nm with subtraction of the OD at a reference wavelength of 620 nm, using a microplate spectrophotometer. Background control contained only culture medium without cells. Cells without treatment were included as control of maximum cell growth.

Cytotoxicity Assay

Flat bottomed 12-well microculture plates were seeded with $5x10^5$ cells in 100 μL/well and grown in DMEM suplemented with 1% FCS in presence of 1/10 diluted sera. As control of unspecific complement cytotoxicity was used sera from non immunized mice. To mesuare specific complement independent cytotoxycity , the immune sera were heated 30 minutes at 56°C. After 24 hours of incubation at 5% of CO2, death cells were measured by FACS using Propidium Iodine. Cells without treatment were included as control of minimum death cell.

Reproductive side effects studies

Female Balb/c mice (n=10) were immunized with ECD-mEGFR/VSSP as previously described, and a control group received PBS/VSSP. After the evaluation of the induction of specific antibodies against ECD-mEGFR, mice were mated with non immunized male mice. The fertility (number of mice completing pregnancy), number of pups, the birth weight of the pups, and the postnatal developmental features such as eyes opening, hair growth and incisor eruption were observed. Statistical differences in the weight of pups 1 day old were determined using Mann-Whitney test.

Statistical Analyses

Equality of variances was analyzed with Bartlett´s test and Bonferroni test was used to analyze normal distribution of data. Differences in DTH between treatment groups were tested by Kruskal Wallis and Dunn´s Multiple Comparisson test. For comparison of individual dilutions of sera in IgG subclasses determination, and differences in the *in vitro* viability assay an Unpaired *t* test was used. The Mann Whitney test was used to assess statistical differences between individual time points in the kinetic of humoral response and

to test significant variations in the reproduction parameters between treated and control animals. A probability value of $p<0.05$ was considered as statistically significant. All statistical tests were two-sided, and conducted using GraphPad Prism version 4.00 Software.

RESULTS

Expression and purification of ECD-mEGFR and ECD-Her1

cDNAs encoding ECD-mEGFR and ECD-Her1 were successfully cloned into pcDNA3 expression vector (Fig. 1A) and transfected in HEK293 cells as was described in Material and Methods. Expression of the soluble recombinant proteins by the stable HEK293 transfectants was checked by lectin-agarose precipitation, for ECD-mEGFR or immunoprecipiation with MAb R3 for ECD-Her1. A band of protein was displayed at the expected level of 105 kDa but not for mock transfection in each case, determined by SDS-PAGE. In the figure 1B are showed the results for the ECD-mEGFR. The identity of ECD-mEGFR was confirmed when transfectant 293 culture supernatant was purified by affinity chromatography with hrEGF-EAH sepharose, and the protein at 105 kDa was again displayed by SDS-PAGE (Fig. 1C). The purity was 98% after only one step of purification, measured by a densitometer (data not shown).

mEGFR is expressed in thymus

As has been previously reported from studies with rat cell lines (30) and human thymus (31), we demonstrated by RT-PCR that EGFR is present in the thymus of mice. Figure 2 shows the corresponding band of cDNA of EGFR when thymus from C57BL/6

and Balb/c mice was analyzed, but not from murine thymoma EL4, used as negative control.

ECD-mEGFR immunization induces DTH response

The generation of a DTH response was used in our study as a primary evaluation of the ability of a vaccine composition containing the ECD-mEGFR emulsified in FA to immunize mice. For that reason, mice were subcutaneously injected with ECD-mEGFR/FA and then sensitized with 50 μg of ECD-mEGFR. After 48 hours, the swelling of the mice foot was measured. Mice showed a foot inflammation statistically higher than negative control group (Dunn´s Multiple Comparison Test, $P<0.05$), but similar to the group of mice immunized with KLH/FA, a foreign protein for mice ($P>0.05$) (Fig. 3).

ECD-mEGFR immunization induces a strong and long lasting specific humoral response

To explore the humoral response caused by vaccine compositions containing the ECD-mEGFR, C57BL/6 mice were immunized four times biweekly with 50 μg of ECD-mEGFR in two different oily adjuvants: FA, used as reference adjuvant and VSSP which could be used in humans. All mice developed a very high IgG antibody response against the immunizing protein, and increasing titers were obtained with successive immunizations using both adjuvants (Fig. 4). But the use of VSSP induced antibodies titers statistically higher that FA for each collection day (Mann Whitney test, $P<0.05$). As is shown in the Table 1, 8 of 10 mice (80%) immunized with ECD-mEGFR/VSSP produced titers above 1/40000 on day 56, reaching up to 1/160000 whilst only 2 of 10 mice (20%) had titers above 1/40000 in the group immunized with ECD-mEGFR/FA. After one year, without

antigen recall, the IgG titers decreased in both groups, but an appreciable response was detected in 100% of tested mice (n=5) with titers between 1/1000 and 1/100 (data not shown).

Immunization of Balb/c mice with ECD-mEGFR in FA or VSSP produced high IgG titers in 100% of mice, very similar to the obtained with C57Bl/6 mice (data not shown).

Immunization with ECD-mEGFR induces Th1 associated isotype

Consistent with previous results demonstrating that complete FA induces a TH1 response to the antigen (32), mice immunized with ECD-mEGFR/FA produced elevated levels of IgG2a, IgG2b and IgG1, determined by ELISA with the sera collected on day 21. As is shown in Figure 5 no different levels of IgG2a were found using FA and VSSP in the immunizations (Unpaired *t* test, $P>0.05$), whilst the use of VSSP induced statistically higher levels than FA of the IgG2b ($P<0.05$) and IgG1 subclasses ($P<0.05$).

Immunization with ECD-Her1/VSSP induces specific B cell clones which have low cross-reaction with ECD-mEGFR.

In order to evaluate if immunization with the ECD of heterologous EGFR could induce a humoral immune response which recognize self EGFR, we immunized C57BL/6 mice with 50 μg of ECD-Her1 in FA or VSSP. All immunized mice, with both adjuvants, developed high IgG antibody titers against the human protein which could reach beyond 1/320 000 titer on day 56 (data not shown). The sera cross-reaction with ECD-mEGFR from day 21, one week after the second immunization, was evaluated by ELISA. The heterologous sera, which had a high recognition of human protein, showed an evident but low cross-reaction with murine protein (Fig. 6A). In fact, that recognition was much lower than sera from mice

immunized with ECD-mEGFR (data not shown). The low cross-reaction with ECD-mEGFR was confirmed when the induction of ECD-Her1 specific B cell clones in LN of immunized mice was assayed by ELISPOT. Specific ECD-Her1 spot forming cells (SFC), 62 IgG secreting SFC/ 10^6 LN cells, and 6 IgM secreting SFC/ 10^6 LN cells were found, but only 21 IgG secreting SFC/10^6 LN cells cross-reacted with ECD-mEGFR (Fig. 6B).

Immune sera recognizes full length EGFR by FACS

To check if the immunizations with a truncated EGFR affected the recognition of full length EGFR in its native conformation on the cell surface, some EGFR+ cells were analyzed by FACS. EAT (33) and 3LL-D122 murine cell lines were recognized by sera from mice immunized with ECD-mEGFR/VSSP (Fig. 7A). Besides, sera from mice immunized with ECD-Her1/VSSP recognized A431 and H125 cell lines (Fig. 7B). The sera from control mice immunized with PBS/VSSP did not recognize neither murine nor human EGFR+ cells.

Immune sera inhibit EGFR+ tumor cells growth and have cytotoxic effect

In order to determine whether immunization with ECD-mEGFR/VSSP or ECD-Her1/VSSP has an effect on the growth of murine or human tumor cells *in vitro,* respectively, the MTT-viability assay was performed. Incubation of 3LL-D122 or H125 cells with sera from mice immunized with ECD-mEGFR/VSSP or ECD-Her1/VSSP respectively, showed a statistically lower number of viable cells with respect to treatment with pre-immune sera after 48 hours, and this effect was sera dilution dependent (Unpaired t test, $P<0.05$) (Fig. 8A). In addition, the *in vitro* cytotoxicity of immune sera over EGFR+ cells was evaluated treating cells during 24 hours with complement inactivated sera, and

then stained with Propidium Iodine. FACS analysis showed that treatment of 3LL-D122 cells with immune sera produced 55,83% of death cells, suggesting that a complement independent cytotoxicity mechanism is operating (Fig. 9A). In parallel, H125 cells were treated with sera from mice immunized with ECD-Her1/VSSP, and the effect in the cells growth and cytotoxicity were evaluated. The immune sera decreased the number of viable cells, compared with preimmune sera (Fig. 8B), and showed a cytotoxic effect by FACS (Fig. 9B).

Immunization of ECD-mEGFR in VSSP produces no evident reproductive side effects

To study the potential side effects that self immunization might cause in humans, we have examined the possible toxicity generated in female mice immunized with ECD-mEGFR in VSSP. These were then mated and their progeny studied. In our studies the rates of pregnancies were 5/10 for immunized mice and 3/10 for the control group. The median number of pups per litter was 6 (range 5 to 7) in both groups. The features observed in pups did not differ between both groups, without statistical differences ($p>0.05$) in the weight of newborns, hair growth, eyes opening and incisor appearance, measured in days after birth (d.a.b.), as shown in Table 2. On the other hand, a group of mice were observed for one year after the immunization, and the vitality, temperature, and food intake were completely normal, without changes in the functional hepatic parameters when compared with non immunized mice (data not shown).

DISCUSSION

Despite the wide expression of EGFR in the organisms, it can be considered a tumor-associated antigen (TAA) due to its overexpression in many tumors of epithelial

origin (34), its implication in the tumor growth, and correlation with bad prognosis (12). For that reason the EGFR has become an attractive target for cancer therapy and many attempts are being made using this molecule as target for passive therapy with tyrosine kinase inhibitors and monoclonal antibodies (35-37). More recently a DNA vaccine based on xenogenic EGFR and a vaccine based in dendritic cells pulsed with ECD of self EGFR (20-[3]) has been proposed. Our results demonstrate that the extracellular domain of murine EGFR in an appropriate oily adjuvant is very immunogenic in mice. Thus, vaccination using the ECD-EGFR protein can be considered as an attractive strategy for cancer treatment.

The presence of tolerance to TAA represents a significant challenge to effective immunotherapy of human cancer and successful vaccine strategies must circumvent this tolerance. Some recent literature demonstrates that it is possible to break self tolerance, the self recognition being a physiological phenomenon (38). But owing to the fact that EGFR is widely represented in the organism, circumventing the tolerance to this self antigen could initially seem difficult. As a primary evaluation of the immunogenicity of the ECD-mEGFR, without any conjugation to a carrier protein, the induction of DTH was measured in our studies. For that purpose, we emulsified the protein in FA, which is known to be one of the stronger TH1 adjuvants. The ECD-mEGFR proved to be a very immunogenic protein which produced severe inflammations in the mouse foot like KLH, a foreign protein for mice. Once we had this evidence, we explored the quality of a humoral response induced by this vaccine composition, and we introduced the VSSP in our studies, an adjuvant that could be used in a potential cancer vaccine. It is known today that adjuvants

are an important component of vaccine formulations, providing the necessary red flag signals to initiate an effective immune response.

Immunization of mice with ECD-mEGFR in FA and VSSP induced a specific immune response with high IgG antibody titers, which increased with successive immunizations, indicating the presence of a mature immune response. The use of VSSP produced higher levels of IgG than FA, indicating the potential of this adjuvant. In both cases the subclass distribution was characterized by the presence of IgG2a and IgG2b isotypes, as an indirect indication of the differentiation of TH response into TH1, and high levels of IgG1 were also induced. But higher levels of IgG2b and IgG1 were obtained using VSSP as a confirmation of the effectively of this adjuvant. It is known that self immunotolerance can be breached, but the reactivity towards self-proteins is limited. Indeed, early attempts using another member of the EGFR family, HER2, succeeded when monkeys were immunized with HER2-ECD in Detox adjuvant after 6 immunizations. However, IgG titers never reached 1/10000 (39), which is much lower than the IgG titers we induced with ECD-mEGFR in FA or VSSP. Besides, Disis et al (40) have shown that a neu-peptide-based vaccine, but not a whole-protein vaccine, can elicit humoral and cellular responses in rats. EGFR is a tolerated self-antigen for which central thymus deletion could be occurring. The induction of central T-cell tolerance requires the presence of autoantigens in the thymus (41,42), and the expression of EGFR in thymus, early reported for humans (12) and rats (30), was confirmed in our lab, detecting EGFR by RT-PCR in thymus of Balb/c and C57Bl/6 mice. No presence of natural autoantibodies against EGFR has been reported in normal people, which we also observed in mice, when sera from non-immunized mice were tested by ELISA and FACS to recognize ECD-mEGFR or EGFR+

murine cells. Mounting evidence shows that the tolerance to EGFR can be breached in certain cancer patients and discrete natural humoral responses against the EGFR have been detected (43). Similar observations have been reported for HER2, where patients with breast cancer show HER2-specific response in the form of antibody and CTL responses (44) which are not sufficient to prevent tumor progression. But, the endogenous immunity to HER2 is only present in a minority of patients which overexpress HER2 and, if detectable, is of low magnitude (45). Vaccines targeting these two targets, HER1 and HER2, therefore must be focused on circumventing tolerance generating significant levels of specific antibodies and T cells, and only boosting the already present immunity in a minority of patients. In fact, HER2 vaccines constructed with synthetic peptides mixed with granulocyte-macrophage colony stimulating factor as adjuvant have been used in clinical trials in patients with breast, ovarian and lung carcinomas which overexpress HER2, and 68% of them developed T cell immunity against self HER2 protein (46).

Today, even when it is clear that self recognition pre-exists or can be induced by immunization, what is not extensively supported by the literature is that self-recognition can be induced by self-immunization. The high immunogenicity of the protein in our studies could be explained by the truncation of the full length EGFR and the context of presentation to the immune system. Truncation of the EGFR could change the immunodominance of the recombinant ECD, inducing the presentation of cryptic or subdominant determinants to the immune system (47). These possible changes do not affect the recognition of the full length protein in its natural conformation in the cells, as was observed by FACS experiments. However, this result contradicts that reported for HER2, in which rats elicit no detectable immune response when they immunized with the

intracellular domain (ICD) of rat neu but elicit CTL and humoral response specific for both human HER2/neu and rat neu when they were immunized with the highly homologous foreign human ICD (48). On the other hand, the use of potent adjuvants in our studies create a TH1 context that could break the "no-response state".

The key to success in any active specific immunotherapy could be a coordinated action of both the humoral and cellular arms of the immune response. Complete FA was used as a reference adjuvant because it is highly effective in inducing both cellular and humoral immunity, and because of its known capacity to induce TH1-dominated immune response (32). While Complete FA may have limited use in humans because of its inflammatory side effects, VSSP adjuvant has some advantages due to its peculiar immunomodulator properties and its possible use in humans. Previous reports show that vaccinating mice with VSSP induces high titers of IgG against GM3, a very poor immunogenic ganglioside, with isotypes related with a Th1 pattern (26), and induce dendritic cell maturation and IL-12 production (49) which is pivotal for the development of TH1 responses (50,51).

Even when the amino acid sequence homology between the ECD of human and murine EGFR is about 87%, the minor differences could be responsible of the high inducible immune response. In fact, the sera from mice immunized with ECD-Her1 recognize with low titers the murine protein. Besides, only the third part of the secreting IgG B cells induced by the vaccination with ECD-EGFR crossreact with ECD-mEGFR. The immune response to the human protein can be directed to the not homology region between these two proteins. That result suggest that a potential vaccine for human cancer patients should be based in the ECD-Her1.

In the present study we found that immune sera from immunization, using VSSP as adjuvant, decreased the growth of EGFR+ tumor cells and had an complement independent cytotoxic effect over those cells. This result shows the quality of the immune response induced by vaccination.

EGFR-targeted therapies are expected to produce side effects related to the induction of autoimmunity. In fact, some side effects as skin rash have been reported for some therapies (52), but important toxic effects have not been found for the majority of the different modalities of therapies which are undergoing clinical trials. As example, the monoclonal antibody THERACIM (humanized R3, CIM, Cuba) has been tested in Phase II head and neck cancer clinical trials in combination with radiotherapy, using 600 mg/cycle, without cases of skin rash. At the same time it has been reported that inhibition of EGFR gene expression is critical for cancerous cell growth but not for normal cells (53). Active immunization, providing a long lasting specific immune response, should be carefully monitored for possible side effects. In our case, we conducted the experiments with ECD-mEGFR/VSSP. Even one year after the last immunization, animals present low titers of ECD-mEGFR specific IgG, but they do not show evident signs of toxicity and functional hepatic parameters behave as in non immunized mice. It has been reported that induction of EGF deficiency in rats affects the development of fetal tissue, but not the adult tissues (54). Considering the role of EGF in the epigenetic regulation of fetal and neonatal development, we studied the effect of EGFR immunity in the fertility of female immunized mice and their progeny, and no marked side effects could be found.

We conclude that ECD of self EGFR is very immunogenic in the context of FA and VSSP. More studies will be conducted to understand more fully the cellular arm of the

induced immune response. Taken together, the immunization approach described here may be an attractive and novel strategy for EGFR+ cancer treatment.

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Figure captions

Figure 1: Construction and functionality of expression vector and purification of ECD-mEGFR: A) The DNA enconding the ECD-mEGFR and ECD-Her1 were inserted into the pcDNA3 expression vector. **B)** ECD-mEGFR/pcDNA3 construct was verified by sequencing and protein expression was detected by precipitation with lectin-agarose from supernatant of HEK293 transfectant, and visualized by 7.5% SDS-PAGE with silver staining. Mock transfection (pcDNA3) was used as control. **C)** Purification and confirmation of identity of the ECD-mEGFR was made by affinity chromatography with hrEGF-EAH sepharose.

Figure 2: EGFR is expressed in the thymus of mice. Thymus from C57/Bl6 and Balb/c mice was analyzed by RT-PCR for the expression of EGFR. Total RNA was extracted from thymus of C57Bl/6 and Balb/c mice or from EL4 tumor used as negative control, using TRIZOL reagent; 1 μg of RNA was reversed-transcribed to cDNA and the specific fragments amplified by PCR. β-actin mRNA served as an internal control.

Figure 3: ECD-mEGFR specific DTH response. DTH response was assayed by immunizing C57Bl/6 mice two times with 50 μg of ECD-mEGFR/FA or 50 μg of KLH/FA as positive control or PBS/FA as negative control. Mice were sensitized 7 days later with the same immunizing antigen for the first two groups or with ECD-mEGFR to the PBS control group. Group treated with ECD-mEGFR showed statistical higher inflammations ($p<0.01$) than PBS control mice, but similar ($p>0.05$) than KLH group. One representative experiment from two independent experiments is shown.

Figure 4: Kinetic of ECD-mEGFR specific humoral response. Mice were immunized with 50 μg of ECD-MEGFR in VSSP or FA, 4 doses biweekly. Antibody titers were assayed by ELISA with the sera collected on days 0, 21, 35 and 56. Data was log transform (1+1/titer) for the graphic. No antibodies were detected before the immunization (day 0), and increased antibodies titers were detected after immunizations, which were statistically higher when VSSP adjuvant was used ($P<0.01$). The arrows represent the immunization days. One representative experiment from three independent experiments is shown.

Figure 5: IgG subclasses induced by immunization. The IgG subclasses induced in C57Bl/6 mice after the immunization with 50 μg of ECD-mEGFR in VSSP or CFA, 2 doses biweekly, was assayed by ELISA with the sera collected on day 21, and diluted 1/10 000. Each point represents mean absorbance of duplicate samples of individual mice (n=5). IgG1 levels were higher ($P<0.05$) in the group immunized with ECD-mEGFR/VSSP as well for IgG2b ($P<0.05$) but not for IgG2a. One representative experiment from three independent experiments is shown.

Figure 6: Immunization with ECD-Her1/VSSP induces B cell response: Mice were immunized with 50 μg of ECD-Her1/VSSP, 2 doses biweekly. **(A)** The specific IgG titters against ECD-Her1 and their cross-reaction with ECD-mEGFR were assayed by ELISA, with the sera collected on day 21. **B)** Specific spot forming cells, secreting IgG and IgM antibodies against ECD-Her1 and their cross-reaction with ECD-mEGFR were measured by ELISPOT. One representative experiment from two independent experiments is shown.

Figure 7: Immune sera recognize EGFR+ tumor cells. A) Sera from mice immunized with ECD-mEGFR/VSSP diluted 1/200 reacted with 3LL-122 and EAT cell lines (black line). **B)** Sera from mice immunized with ECD-Her1/VSSP diluted 1/200 reacted with A431 and H125 cell lines (black line). Sera from mice immunized with PBS/VSSP (gray line) were used as negative control.

Figure 8: Immune sera inhibit EGFR+ tumor cells growth. A) 3LL-D122 cells or **B)** H125 cells were grown in presence of serum from mice immunized with ECD-mEGFR or ECD-Her1 respectively. Preimmune sera were used as negative control. After 48 hr, viability of cells was measured by MTT colorimetric assay. Both ECD-mEGFR and ECD-Her1-immune sera decreased statistically the number of viable cells ($P<0.005$). Each bar represents mean absorbance ± SD of triplicate samples. This experiment is representative of two independent experiments.

Figure 9: Cytotoxic effect of immune sera over EGFR+ cells: A) 3LL-D122 and **B)** H125 cells were incubated 24 hours with inactivated-complement sera from mice immunized with ECD-mEGFR/VSSP or ECD-Her1/VSSP respectively. FACS analysis shows the cytotoxic effect of immune sera. This experiment is representative of two independent experiments.

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